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Relationship between lichen species composition, secondary metabolites and soil pH, organic matter, and grain characteristics in Manitoba

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Abstract: Many lichen secondary metabolites have functions related to the environmental conditions of lichen habitats but few studies have compared soil characteristics with lichen species composition or their secondary products. The goal of this study was to investigate the relationship between soil characteristics with lichen species composition and secondary metabolites. Five locations were chosen in Manitoba, each with five sites (transects), and each transect with five quadrats (1 m × 1 m). All species were collected from each of the quadrats, presence of secondary metabolites was determined by thin layer chromatography, and soil characteristics were examined. The results revealed that rounded sand grains were significantly higher in southeastern Manitoba than in other locations, corresponding to a distinct species composition. Angular grains were significantly higher in northern locations, corresponding to a different group of species. Some of the significant relationships between soil characteristics and secondary metabolites include correlations of atranorin with pH, organic matter, and sand content; fumarprotocetraric acid with organic matter and sand content; and usnic acid with pH and organic matter. A better understanding of the role of lichens with respect to soil characteristics will be important for improving soil stabilization in land reclamation.

Key words: Cladonia, lichen species, organic matter, sand particles, secondary metabolites.

Résumé : Plusieurs métabolites secondaires des lichens exercent des fonctions reliées aux conditions environnementales des habitats des lichens, mais peu d'études ont comparé les caractéristiques des sols et la composition en espèces de lichen ou leurs produits secondaires. Le but de cette étude était d'examiner la relation entre les caractéristiques des sols et la composition en espèces de lichen et les métabolites secondaires. Cinq emplacements ont été choisis au Manitoba, comprenant chacun cinq sites (transects) séparés en cinq quadrants (1 m × 1 m). Toutes les espèces ont été récoltées à partir de chaque quadrant, la présence de métabolites secondaires a été déterminée par chromatographie sur couche mince et les caractéristiques des sols ont été examinées. Les résultats ont révélé que les grains de sable arrondis étaient significativement plus abondants dans le sud-est du Manitoba que dans les autres emplacements, correspondant à une composition distincte en espèces. Les grains de sable angulaires étaient significativement plus abondants dans les emplacements nordiques, correspondant à un groupe différent d'espèces. Certaines des relations significatives entre les caractéristiques des sols et les métabolites secondaires comprennent les corrélations entre l'atranorine et le pH, la matière organique et le contenu en sable, entre l'acide fumarprotocétrarique et la matière organique et le contenu en sable, et entre l'acide usnique et le pH et la matière organique. Une meilleure connaissance du rôle des lichens en ce qui concerne les caractéristiques des sols sera importante pour améliorer la stabilisation des sols dans la restauration des terres. [Traduit par la Rédaction]

Mots-clés : Cladonia, espèce de lichen, matière organique, particule de sable, métabolites secondaires.

Introduction

The effect of environmental conditions on individual lichen species has been shown through studies in which environmental variables were linked to morphological differences (Gilbert 1977; Goffinet and Bayer 1997; Pintado et al. 1997; Baloch et al. 2010; Kotelko and Piercey-Normore 2010; Cornejo and Scheidegger 2013; Muggia et al. 2014). Some of these studies supported the

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optimal defence theory (McKey 1974; Rhoades 1979; Asplund et al. 2010) in which secondary metabolite production was correlated with herbivory, and abiotic variables such as light (McEvoy et al. 2006; Armaleo et al. 2008), pH (Fox and Howlett 2008; Timsina et al. 2013), and humidity (Culberson and Armaleo 1992; Stocker-Wörgötter 2001). However, few studies have examined the relationship between substrate and the lichen phenotype or secondary metabolite production (Brodo 1973; Gilbert 1977). Additionally, the substrate has been examined with respect to water soluble nutrients available for lichen soil crusts (Fischer et al. 2014), substrate moisture in an Alaskan tundra (Williams et al. 1978), and as an important source of nutrients by fungal enzyme activity (Moiseeva 1961). The role of the soil in nutrient and moisture availability corresponded with species distribution (Jun and Rozé 2005) and would be expected to depend to some degree on the extent of direct contact between the soil and the lichen thallus. To our knowledge, no study has examined the relationship between soil characteristics with lichen species composition or secondary metabolite production.

Secondary metabolites produced by lichen-forming fungi comprise diverse groups of metabolites (Elix and Stocker-Wörgötter 2008). A well-studied group includes the polyketides, which are produced by the fungal partner in the acetyl-polymalonyl pathway. A large body of literature is available on polyketide production in non-lichenized fungi (Keller and Hohn 1997; Sanchez et al. 2010; Chen et al. 2014) and it is growing for the lichenized fungi (Stocker-Wörgötter 2001, 2008). While the biosynthesis of some polyketides from non-lichenized fungi is being unravelled (e.g., Chiang et al. 2009, 2010; Campbell and Vereras 2010; Weissman 2015), knowledge of the biosynthesis of the many unique compounds from lichenized fungi is lagging behind, with some progress made in usnic acid (Abdel-Hameed et al. 2016). Better knowledge of the conditions required for culturing the lichen fungus to induce polyketide production would facilitate progress in these studies. Because lichen polyketides are produced in nature, it might be expected that a better understanding of the natural conditions, including the substrate, would facilitate culturing. The potential for the application of polyketides in industry or health related fields has increased the interest in this group of compounds.

Polyketide production has been hypothesized to be associated with slow growth of the fungus. For example, it has been suggested that overwintering stages, reproduction, and other life stages may cause slow growth (Bu'Lock 1961; Calvo et al. 2001; Timsina et al. 2013). Polyketide production may also be regulated by the carbon–nutrient balance (CNB) hypothesis (Bryant et al. 1983; Hyvarinen et al. 2003), which is based on the concept that an imbalance of nutrients will promote production of carbon-based secondary metabolites. The hypothesis suggests that when a plant or lichen is growing in nutrient-poor conditions, the carbon may be shunted into carbon-based secondary metabolites such as polyketides. When the plant or lichen is growing in nutrient-rich and balanced conditions, the carbon may be used in thallus growth rather than production of secondary metabolites. However, the hypothesis is controversial because it represents a complex model with many interacting variables (Koricheva 2002). For example, soil features and community structure may influence the nutrients available to lichens and their growth rates; therefore, they may indirectly influence production of secondary metabolites in lichens.

Some lichens are extreme stress tolerators and able to adapt to harsh conditions on sandy soils in areas where plants are absent, reducing the competition on lichens and allowing them to dominate in these locations (Botting and Fredeen 2006). Jun and Rozé (2005) found a significant relationship between the edaphic parameters (soil pH, water content, nutrient content) and the distribution of species for lichens and bryophytes in the sand dunes of the French Atlantic coast. The content of organic material and carbon also affected the occurrence of some species in sandy areas. For example, the occurrence of some species of *Cladonia*, such as *Cladonia arbuscula*, *Cladonia mitis*, and *Cladonia portentosa*, are favoured by the acidification of the substrate (James et al. 1977), whereas differences in the occurrence of *Cladonia pocillum* and *Cladonia pyxidata* corresponded with the pH of the soil on which they were growing (Kotelko and Piercey-Normore 2010).

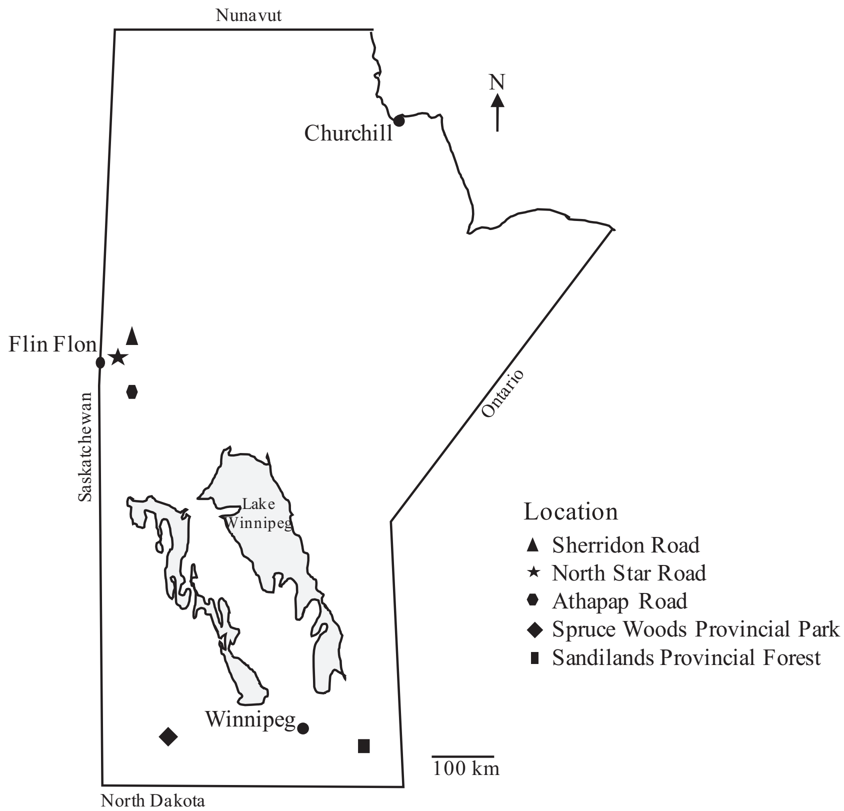
The goal of this study was to investigate the relationship among secondary metabolites, lichen species composition, and soil characteristics in five locations in Manitoba. The soil characteristics were examined to determine which characteristics had the largest influence on species and secondary metabolite occurrence.

Methods

Experimental design and sample collection

Field collections were made in five locations in northern and southern Manitoba (Fig. 1), and were focused in areas with sandy soils (Table 1). The five locations have a continental climate and contain soils that vary in underlying geology (Table 1; Fig. 1). Samples of all species of lichens were collected from each of five quadrats (1 m × 1 m) in each of five sites (transects) in each of five locations (125 quadrats in total). The quadrats were placed every 5 m along a 25 m transect within each site and the upper right corner of the quadrat was placed over a lichen thallus. The percent ground cover of the species recorded as guilds and other biotic (vascular plants, bryophytes, and lichens by general categories) and abiotic (bare rock, exposed soil, and vegetative debris such as twigs, cones, leaves, etc.) factors were recorded by visual estimation to the nearest 5% using a 25 cm × 25 cm grid

Fig. 1. Map of Manitoba, Canada, showing locations of collection sites (transects) in this study: Sandilands Provincial Forest, Spruce Woods Provincial Park, Athapap Road, Sherridon Road, and North Star Road.



placed within the quadrat for estimating coverage. Percent canopy cover was estimated using the same grid and held directly above the quadrat to estimate the amount of light. Specimens were air dried and returned to the lab for identification using methods described by Ahti (2000), Brodo et al. (2001), Hinds and Hinds (2007), and Kowalewska et al. (2008). To determine the secondary metabolites present, thin layer chromatography was performed on all specimens using solvent A (185 mL toluene, 45 mL dioxane, and 5 mL glacial acetic acid) (Orange et al. 2001). Briefly, about 200 uL of acetone was applied to the crushed thallus samples, which were left soaking for 20 min. Acetone extracts were applied to silica-coated glass plates, immersed within solvent A in a thin layer chromatography tank until the solvent front reached the top of the plate. After drying, the plates were observed under long (365 nm) and short (255 nm) wave UV light, sprayed with 10% sulphuric acid, and heated at 80 °C for 20 min. Standard secondary metabolites as positive controls were prepared from herbarium specimens that had the chemicals previously identified for comparison. Known Rf classes and further spot comparisons were made with characteristics reported by Orange et al. (2001). Frequency of occurrence for secondary metabolites was recorded.

Soil characteristics

Two soil samples (multiple representative samples and a single bulk sample) were collected from each of the 125 quadrats (25 in each of 5 locations). Multiple samples were collected from different places in the quadrat and placed within clean plastic bags (about 1 kg) to represent the surface soil in the quadrat (0 to 7 cm depth) and to determine soil characteristics. Bulk density samples were collected from the center of the quadrat using a standard bulk density cylinder (7.62 cm height × 7.62 cm diameter). Organic matter was calculated as loss of carbon on ignition by burning the soil samples at 650 °F for 18 h using a muffle furnace (Barnstead/Thermolyne). Percentage of the main soil components (sand, silt, and clay), and gravel as an additional soil component, were determined by sieving the soil samples with different soil sieves using the Canadian standard sieve sizes (Fisherbrand US Standard Brass Test Sieves; sieve number 10 (2.00 mm opening) to collect the silt and clay grains, sieve numbers 35 (0.50 mm opening) and 100 (0.149 mm opening) to collect the fine and coarse sand, respectively, and sieve number 200 (0.074 mm opening) for rocks and pebbles). Shape of the sand grains was also determined and divided into two main categories (angular and rounded) (Arculus et al. 2015) for 1 sample from each of 125 quadrats using a dissecting microscope (Leica MZ 6) at 10× magnification. The grain shape was determined from a soil analysis kit (Forestry Suppliers, Inc., Mississippi, USA). A distilled water mixture with soil was prepared for measuring the pH of the soil using a pH meter

Table 1. General characteristics of the locations of collection sites (transects) including latitude and longitude, prevalent parent bedrock material, maximum and minimum yearly temperatures, and location descriptions with dominant vegetation.

Characteristics	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Latitude and longitude	54°77′72.9″N 101°47′37.5″W	54°69′75.4″N 101°68′12.3″W	54°51′58.8″N 101°53′19″	49°43′45.4″N 99°17′29.2″W	49°44′31.9″N 96°39′22.8″W
Prevalent parent bedrock material	Granite	Gneiss	Calcaric regosols	Aeolian regosols	Glacial till regosols
Max/min yearly temperature (°C)	23.8/−24.7	23.8/−24.7	24/−28.9	26/−24.3	26/−23.2
Location description and dominant plant species	Precambrian Shield. Patches of soil in crevices of bedrock and among outcrops. Aspen, birch, white spruce, Jack pine, willow, and alder.	Precambrian Shield. Patches of soil in crevices of bedrock and among outcrops. Aspen, birch, white spruce, Jack pine, willow, and alder.	Shallow regosol on limestone bedrock. Mixed forest with aspen, white spruce, fir, white birch, and alder.	Active and stabilized sand dunes. Grasses and bluffs of white spruce, aspen, and juniper in depressions. Oaks on high ridges.	Rolling forest and sandy till. Jack pine, aspen, and white spruce interspersed with low-lying black spruce stands.

(VWR sypHony SB70P). The pH measures were performed according to standard methods (Benton and Jones 1999).

Statistical analyses

Principle coordinates analysis (PCoA; Legendre and Legendre 1998) was used to test the similarities among lichen species composition using the R package "vegan" (Oksanen et al. 2015). The studied species were scored as present or absent. To examine whether soil characteristics influenced secondary metabolite frequency and species compositions, redundancy analyses were performed using the "rda" function in the R package "vegan". Monte Carlo permutation tests were performed to test the significance of redundancy analysis. The frequency of occurrence of secondary metabolites was tested by recording the number of quadrats in which each secondary metabolite was found. The soil pH levels were divided into two groups: high acidic soil (pH ranging from 4.2 to 5.7) and neutral to slightly acidic soil (pH ranging from 6.1 to 7.4) to compare frequency of secondary metabolites and lichen species. Significance tests for soil characteristics, biotic and abiotic factors, species frequency, and secondary metabolite frequency were performed using ANOVA by the JMP program 12.1.0 (64 bit), and comparison of the means was done by Tukey's honestly significant difference test. The frequency of secondary metabolites and species occurrence (number of quadrats in which each secondary metabolite or species was detected) was examined with location as a categorical variable. To test the relationship between soil features (organic matter, pH, and sand content) and occurrence of secondary metabolites as continuous variables, a correlation was performed using the JMP program 12.1.1. Statistical analyses were performed with the R program (R Core Team 2013), using a significance level of α = 0.05 unless otherwise indicated.

Results

Soil characteristics for each of the five locations are described in Table 2. Gravel content was highest in the Sandilands Provincial Forest (18.2% ± 3.2%). The sand content in Spruce Woods Provincial Park (92.6% ± 0.5%) was significantly higher than that of the other four locations. The finest sand grains were in the Sandilands Provincial Forest and Spruce Woods Provincial Park, and the coarsest sand grains were in North Star and Athapap. The percent of rounded sand grains was significantly higher in Sandilands Provincial Forest (82.2% ± 2.1%) than other locations whereas angular grains were more abundant in North Star than other locations (84.9% ± 2.2% though not significantly different from Sherridon). The highest rounded/angular (R/A) ratio was in Sandilands Provincial Forest. Clay and silt levels were significantly higher in Athapap (35.8% ± 2.0%) than in other locations. Organic matter was significantly higher in Sherridon and Athapap than the other locations (28.6% ± 5.7% and 27.%9 ± 5.6%, respectively). The pH level was significantly higher

Table 2. Soil characteristics in five sites (transects) across the five locations in this study.

Soil characteristics	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Gravel (p = 0.0001)	11.7±1.7ab	9.8±0.9b	8.9±1.1b	6.4±0.5b	**18.2±3.2a**
Sand (p < 0.0001)	65.8±3.9c	60.2±1.6cd	54.8±2.6d	**92.6±0.5a**	78.7±3.2b
Fine sand (p < 0.0001)	61.6±3.8b	39.2±1.4c	46.3±0.8c	**89.3±1.4a**	**87.1±1.7a**
Coarse sand (p < 0.0001)	38.4±3.8b	**60.2±1.4a**	**53.6±0.8a**	10.7±1.4c	12.9±1.7c
Rounded grain (R) (p < 0.0001)	28.7±4.0d	15.2±2.1e	57.6±3.3b	40.0±1.2c	**82.8±2.1a**
Angular grain (A) (p < 0.0001)	71.3±5.4ab	**84.9±2.2a**	42.5±4.6c	60.1±1.0b	17.1±3.4d
R/A ratio (p < 0.0001)	0.62±0.2b	0.19±0.03b	1.77±0.2b	0.68±0.03b	**8.22±1.6a**
Clay and silt (p < 0.0001)	18.2±3.4c	27.3±1.7b	**35.8±2.0a**	0.8±0.1d	2.7±0.4d
Organic matter (p = 0.001)	**28.6±5.7a**	17.9±3.5b	**27.9±5.6a**	1.7±0.3c	5.4±1.1c
pH level (p = 0.001)	5.54±0.4bc	4.98±0.3c	6.62±0.1ab	**7.00±0.2a**	6.38±0.2ab

Note: Values are mean ± SE derived from five 1 m^2 quadrats from each of five transects for each of the five locations. R/A ratios are calculated as mean ± SE over the individual values of R/A for the 25 plots sampled in each of the locations. Organic matter values are the percentage of total mass). Bold numbers represent significant values at the given probability levels in the first column for each soil characteristic. Lowercase letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

in Spruce Woods Provincial Park than in North Star but there was no significant difference between the other three locations (Table 2).

The percent canopy and ground covers for the quadrats in each location are shown in Table 3. Briefly, Sherridon, and Athapap had a significantly higher percentage of canopy cover compared with Sandilands Provincial Forest and Spruce Woods Provincial Park. North Star showed a significantly higher percentage of ground cover of lichens than Athapap and Sandilands (Table 3). Bryophyte ground cover was significantly higher in Athapap. The cup-forming lichens were significantly higher in Spruce Woods Provincial Park (except North Star), whereas mat-forming lichens were significantly higher in Sherridon than other locations. *Stereocaulon* and crustose lichens were significantly higher in North Star than the other locations (except Sherridon), the red-fruited lichens were significantly higher in Athapap, but the foliose lichens showed no significant differences among locations. Other factors analysed included vegetative debris, which was high in Sherridon, exposed soil, which was high in Sandilands Provincial Forest, and bare rock, which was high in Sherridon and North Star.

Forty-six lichen species are reported in this study with greater than 10% frequency of occurrence (Table 4). Four species, *Cladonia arbuscula*, *Cladonia chlorophaea, Cladonia gracilis* subsp. *turbinata*, and *Cladonia pyxidata* were the most frequently occurring species being present in 21 or 22 out of 25 total sites (transects) and were present in all five locations. Three species (*Cladonia humilis*, *Cladonia magyarica*, and *Cladonia sulphurina*) were found in one of the five locations (Table 4).

Secondary metabolites with more than 20% frequency of occurrence among the five locations are shown in Table 5. Zeorin, barbatic acid, and usnic acid were significantly high in Sherridon and North Star. Squamatic acid and thamnolic acid were significantly high in North Star. The presence of atranorin was significantly high in Spruce Woods Provincial Park. Fumarprotocetraric acid and perlatolic acid were recorded in high frequency in all five locations and there were no significant differences among them.

The relationship between location and each of the soil characteristics is shown in Fig. 2. In this PCoA, the horizontal axis explains 51.4% of the variation in the data, and the vertical axis accounts for an additional 21.6%. Spruce Woods Provincial Park clustered at the positive end of the horizontal axis with higher sand content and fine sand, whereas Athapap, North Star, and Sherridon clustered at the negative end of the horizontal axis. Sandilands Provincial Forest clustered at the positive end of the horizontal axis with rounded sand grains. The PCoA showed three main groups (Sandilands Provincial Forest, North Star, and Spruce Woods Provincial Park) that were separated based on the soil characteristics (Fig. 2). Athapap and Sherridon had more variable soil characteristics.

A highly significant relationship between the secondary metabolites and the soil characteristics is shown by a Redundancy Analysis (Monte Carlo permutation test, $p = 0.001$) (Fig. 3). The RDA axis 1 accounted for 62.2% of the variation in the data and axis 2 accounted for an additional 22.8% of the variation. Some of the trends show that fumarprotocetraric acid and atranorin were more commonly produced when lichens were growing in sandy fine soils. Grayanic acid, thamnolic acid and merochlorophaeic acid were weakly associated with more neutral pH levels (Fig. 3). Usnic acid, zeorin, and barbatic acid were associated with angular sand grains (Fig. 3). The sand content was negatively correlated with organic matter (Pearson's product-moment correlation $r = -0.89\%$, $p = 0.001$).

A significant relationship was found between species and soil characteristics (Fig. 4). The RDA axis 1 accounts for 44.81% of the variation and axis 2 accounts for an additional 19.93% of the variation in the data. The trends show that *Peltigera rufescens* was associated with rounded sand grains whereas *Cladonia deformis*, *Cladonia phyllophora*, *Cladonia stellaris*, *Stereocaulon tomentosum*, and *Vulpicida pinastri* were more associated with the angular sand grains. It also suggested that *Cladonia cariosa* is associated with soil

Table 3. Mean ± SE percentage of quadrat cover including canopy, vascular plants, vegetative debris, bryophytes, lichens (total and by general categories), unvegetated soil, and bare rock over five 1 m² quadrats for each of five sites (transects) in each of the locations.

Location cover	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Canopy ($p < 0.0001$)	**36±7.2a**	28±5.7ab	**34.4±6.1a**	3.8±2.5c	11.6±4bc
Vascular plants ($p = 0.1000$)	4.9±1.4a	15.1±3.8a	17.1±3.3a	10.6±3a	12.6±4.3a
Vegetative debris ($p = 0.0030$)	**26.5±5.8a**	14.4±4.1ab	11.8±2.4b	7.0±1.9b	11.2±2.5b
Bryophytes ($p < 0.0010$)	10.0±2.4b	2.0±0.6b	**29.0±5.4a**	6.4±2.4b	7.3±1.7b
Lichens, total ($p = 0.0070$)	49.8±4.8ab	**56.64±5.9a**	35.8±4.9b	46.3±4.3ab	34±4.5b
Cup lichens ($p < 0.0001$)	18.7±5b	30.6±5ab	16.4±4.5b	**44.3±4.4a**	20.4±3.3b
Red-fruited lichens ($p < 0.0001$)	0.28±0.09b	0.44±0.1b	**14.1±4.4a**	0.2±0.08b	0.42±0.9b
Crustose lichens ($p < 0.0001$)	0.00b	**13.8±3.7a**	1.1±0.6b	0.00b	0.00b
Stereocaulon spp. ($p < 0.0001$)	1.1±0.6ab	**3.4±1.3a**	0.2±0.13b	0.00b	0.00b
Mat-forming lichens ($p < 0.0001$)	**29.3±4.8a**	6.7±2.3b	1.3±0.6b	1.3±1b	11.2±3.6b
Foliose lichens ($p = 0.052$)	0.16±0.07a	1.3±0.67a	2.7±1a	0.5±0.4a	2±0.8a
Exposed soil ($p = 0.0005$)	0.12±0.07b	1.8±0.8b	2.6±1.2b	4.6±1.2ab	**10.3±3.1a**
Bare rock ($p = 0.0011$)	**5.4±0.6a**	**5.1±1.4a**	0.72±0.6ab	0.00b	0.00b

Note: Bold numbers represent significant values at the given probabilities for each cover characteristic. Lowercase letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

pH levels and *Cladonia humilis* and *Cladonia magyarica* are associated with fine sand and total amount of sand (Fig. 4).

The correlation between soil characteristics and secondary metabolites showed strong relationships. There were strong negative correlations between zeorin, usnic acid, barbatic acid and squamatic acid with pH of the soil but the correlation between atranorin and pH was strongly positive (Table 6). The correlation with organic matter was strongly positive with zeorin and usnic acid, but it was strongly negative with atranorin and fumarprotocetraric acid. The correlations between percent sand content and each of atranorin and fumarprotocetraric acid were strongly positive (Table 6).

The correlation between soil characteristics and species occurrence also showed strong relationships (Table 7). Some species (*Cladonia deformis*, *Cladonia amaurocraea*, *Cladonia gracilis* subsp. *turbinata*, *Cladonia stellaris*, *S. tomentosum*, and *V. pinastri*) showed a strong negative relationship with pH. A strong positive relationship was present between two species (*Cladonia humilis*, *Cladonia magyarica*) and the percent sand content in soil (Table 7).

Discussion

Soil characteristics may explain some species distributions

The significant differences among the five locations were explained by differences in the soil characteristics and partially by species composition. This finding suggests that some species may not rely on the substrate for nutrients and moisture and have a wide tolerance for substrate characteristics whereas other species may be linked to soil characteristics. For example, *P. rufescens*, *Cladonia cariosa*, *Cladonia humilis*, *Cladonia scabriuscula*, and *Cladonia magyarica* segregated with rounded sand grains (Fig. 2 and Table 2). Furthermore, *Arctoparmelia centrifuga*, *S. tomentosum*, *Cladonia deformis*, *Cladonia stellaris*, *Cladonia uncialis*, and *Cladonia amaurocraea* correlated more strongly with angular sand grains. The angular shape of the sand grains may minimize the spaces between sand grains by fitting together but the round shape may allow for more space between grains and, therefore, more water and air movement between grains (Lipiec et al. 2016). The shape of sand grains has been shown to affect the compaction, compressibility, and shear stress of soils and, therefore, also the aeration, water holding capacity, and temperature regulation (Horn 2011; de Bono and McDowell 2015). The thick mat of rhizines diagnostic of *P. rufescens* and the persistent basal squamules in *Cladonia cariosa*, *Cladonia humilis*, and *Cladonia magyarica* would help to stabilize sand better than tall podetia with disintegrating basal squamules such as *Cladonia gracilis* subsp. *turbinata*, *Cladonia cornuta*, *Cladonia amaurocraea*, or *Cladonia uncialis*. This hypothesis would need further testing.

Cladonia phyllophora and *Cladonia gracilis* subsp. *turbinata* are common boreal species (Brodo et al. 2001) that look very similar and were both present in all five locations, suggesting they may be tolerant of a wide range of soil conditions. They may receive their nutrients from a combination of the soil (especially when they are young with squamules attached directly to the soil) and the atmosphere (especially as they age as the squamules disintegrate). *Cladonia phyllophora* has no cortex near the top of a tall podetium and a black mottled base whereas *Cladonia gracilis* subsp. *turbinata* has a smooth cortex giving it an olive colour throughout the length of a tall podetium. The similar morphological features in these species, where both species produce tall podetia and the primary basal squamules disintegrate with time, suggests that the direct connection with the soil by the primary squamules may be severed after the podetia have been produced. It would stand to reason that after squamule disintegration, the colony of tall podetia must rely predominantly on an atmospheric source of nutrients

Table 4. Frequency of occurrence (mean ± SE) of lichen species[a] over 25 1 m^2 quadrats per site (transect) in each of the five locations.

	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Arctoparmelia centrifuga ($p < 0.0001$)	0.00c	**0.64±0.09a**	0.36±0.09b	0.000c	0.00c
Cladonia amaurocraea ($p < 0.0001$)	**0.40±0.10a**	**0.24±0.08a**	0.00b	0.00b	0.00b
Cladonia arbuscula ($p < 0.0001$)	**1.00±0.0a**	0.64±0.09b	0.44±0.1bc	0.16±0.08c	0.52±0.10bc
Cladonia cariosa ($p < 0.0001$)	0.00b	0.00b	**0.56±0.10a**	**0.36±0.09a**	0.04±0.04b
Cladonia chlorophaea ($p = 0.0196$)	0.52±0.10ab	**0.68±0.09a**	**0.68±0.09a**	0.28±0.09b	0.44±0.10ab
Cladonia cornuta ($p = 0.0450$)	**0.48±0.10a**	0.28±0.09ab	0.12±0.06b	0.32±0.09ab	0.28±0.09ab
Cladonia crispata ($p = 0.0650$)	0.12±0.06ab	0.16±0.07a	0.00a	0.04±0.04a	0.00a
Cladonia cristatella ($p = 0.0010$)	0.24±0.08ab	**0.44±0.10a**	**0.04±0.04a**	0.04±0.04b	0.28±0.09ab
Cladonia decorticata ($p = 0.0494$)	0.00a	0.04±0.04a	0.00a	0.12±0.07a	0.04±0.04a
Cladonia deformis ($p < 0.0001$)	**0.40±0.10a**	**0.56±0.10a**	0.00b	0.00b	0.00b
Cladonia gracilis subsp. *turbinata* ($p < 0.0001$)	**0.76±0.08a**	**0.68±0.09a**	**0.52±0.10a**	0.12±0.06b	**0.48±0.10a**
Cladonia grayi ($p = 0.3144$)	0.12±0.06a	0.04±0.04a	0.00a	0.04±0.04a	0.12±0.06a
Cladonia humilis ($p < 0.0001$)	0.00b	0.00b	0.00b	**0.44±0.10a**	0.000b
Cladonia magyarica ($p < 0.0001$)	0.00b	0.00b	0.00b	**0.96±0.04a**	0.000b
Cladonia merochlorophaea ($p = 0.038$)	0.16±0.07a	0.00a	0.00a	0.00a	0.12±0.07a
Cladonia multiformis ($p = 0.0008$)	0.00b	0.08±0.05b	0.16±0.07ab	0.00b	**0.32±0.09a**
Cladonia phyllophora ($p = 0.0331$)	0.20±0.08ab	0.16±0.07ab	0.08±0.05b	0.04±0.04b	**0.28±0.09a**
Cladonia pocillum ($p = 0.0660$)	0.00a	0.00a	0.04±0.04a	0.12±0.07a	0.00a
Cladonia pyxidata ($p = 0.0004$)	0.16±0.07b	**0.64±0.09a**	**0.60±0.1a**	0.36±0.09ab	**0.68±0.09a**
Cladonia rangiferina ($p < 0.0001$)	**0.44±0.10a**	0.16±0.07abc	0.00c	0.04±0.04bc	0.32±0.09ab
Cladonia scabriuscula ($p = 0.0024$)	0.00b	0.04±0.04ab	0.04±0.04ab	**0.28±0.09a**	0.24±0.08ab
Cladonia stellaris ($p < 0.0001$)	**0.44±0.10a**	**0.28±0.09a**	0.00b	0.00b	0.00b
Cladonia stygia ($p = 0.1876$)	0.12±0.10a	0.04±0.06a	0.00a	0.00a	0.04±0.04a
Cladonia sulphurina ($p = 0.0138$)	0.00b	**0.12±0.06a**	0.00b	0.00b	0.00b
Cladonia uncialis ($p < 0.0001$)	**0.40±0.10a**	0.12±0.06b	0.00b	0.00b	0.00b
Cladonia verticillata ($p = 0.0494$)	0.04±0.04a	0.00a	0.00a	0.04±0.04a	0.16±0.07a
Peltigera rufescens ($p < 0.0001$)	0.08±0.05b	0.04±0.04b	**0.56±0.10a**	0.20±0.08b	0.28±0.09ab
Stereocaulon tomentosum ($p < 0.0001$)	0.04±0.04b	**0.32±0.09a**	0.00b	0.000b	0.00c
Umbilicaria deusta ($p < 0.0001$)	0.04±0.04b	**0.28±0.09a**	0.00b	0.000b	0.00c
Vulpicida pinastri ($p < 0.0001$)	0.28±0.09ab	**0.40±0.10a**	0.08±0.05bc	0.00c	0.00b
Xanthoparmelia cumberlandia ($p = 0.0365$)	0.04±0.04ab	**0.20±0.08a**	0.04±0.04ab	0.00b	0.04±0.04c

Note: Bold numbers are significant at the given probabilities for each species as shown in the first column. Lowercase letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

[a]Only those species with >10% frequency of occurrence are included in the table. *Cetraria islandica*, *Cladonia acuminata*, *Cladonia borealis*, *Cladonia botrytes*, *Cladonia cenotea*, *Cladonia coniocraea*, *Cladonia fimbriata*, *Cladonia gracilis* subsp. *gracilis*, *Cladonia mitis*, *Cladonia pleurota*, *Cladonia subulata*, *Nephroma helveticum, Parmelia saxatilis, Stereocaulon grande*, and *Umbilicaria muehlenbergii* were found at frequencies <10% and are not included in the table.

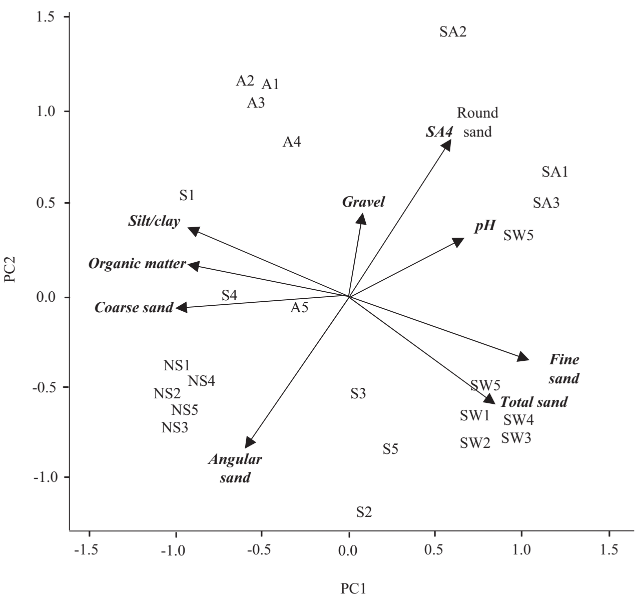
Table 5. Frequency of occurrence (mean ± SE) of secondary metabolites[a] over 25 1 m^2 quadrats per site (transect) for each of five locations in Manitoba.

Lichen acids	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Zeorin ($p < 0.0001$)	**0.48±0.1a**	**0.68±0.09a**	0.00b	0.00b	0.00b
Barbatic acid ($p < 0.0001$)	**0.52±0.1a**	**0.48±0.1a**	0.04±0.04b	0.04±0.04b	0.32±0.09ab
Usnic acid ($p < 0.0001$)	**0.96±0.04a**	**0.92±0.05a**	0.2±0.08c	0.2±0.08c	0.6±0.1b
Thamnolic acid ($p = 0.0138$)	0.00b	**0.12±0.06a**	0.00b	0.00b	0.00b
Squamatic acid ($p = 0.0042$)	0.2±0.08ab	**0.28±0.09a**	0.04±0.04b	0.00b	0.04±0.04b
Perlatolic acid ($p = 0.2757$)	0.16±0.07a	0.24±0.08a	0.04±0.04a	0.16±0.07a	0.08±0.07a
Fumarprotocetraric acid ($p = 0.2479$)	1.00±0.00a	0.92±0.05a	0.96±0.04a	1.00±0.00a	1.00±0.00a
Atranorin ($p < 0.0001$)	0.36±0.08bc	0.32±0.08c	0.68±0.08ab	**0.96±0.08a**	0.4±0.08bc
Grayanic acid ($p = 0.3144$)	0.12±0.06a	0.04±0.04a	0.00a	0.04±0.04a	0.12±0.06a
Merochlorophaeic acid ($p = 0.016$)	0.16±0.07a	0.00a	0.00a	0.04±0.04a	0.2±0.08a

Note: Bold numbers are significant at the given probabilities for each species as listed in the first column. Lowercase letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

[a]Only those metabolites with >20% occurrence are included in the table. Metabolites with <20% occurrence include didymic, gyrophoric, stictic, norstictic, psoromic, salazinic, pinastric, lobaric, alectoronic, and vulpinic acids and are not included in the table.

Fig. 2. Principle coordinates analysis relating soil characteristics including gravel, sand (total, angular fraction, rounded fraction, and grain size), silt and clay, organic matter, and pH in each of the five sites (transects) in each of five locations (NS, North Star Road; S, Sherridon Road; A, Athapap Road; SW, Spruce Woods Provincial Park; SA, Sandilands Provincial Forest). The horizontal axis explains 51.4% of the variation in the data and the vertical axis explains 21.6% of the variation.



and moisture or splash from the substrate or adjacent mosses. Few studies have been conducted on the source of nutrients; however, the substrate has been shown to be correlated with lichen growth for water-soluble nutrients in lichen soil crusts (Fischer et al. 2014), in an Alaskan tundra (Williams et al. 1978), and an important source of nutrients through enzyme activity (Moiseeva 1961). Whereas early squamule growth of these species may penetrate the soil particles and offer some stabilization properties for disturbed soils, the later podetial growth may be more independent of the soil characteristics.

High levels of organic matter may reduce the pH level of the soil, making the soil more acidic (Sparks 2003). Acidic soils seem to be more favourable for *Cladonia amaurocraea*, *Cladonia arbuscula*, and *Cladonia deformis*, whereas *Cladonia cariosa*, *Cladonia pocillum*, and *Cladonia magyarica* are known to grow in calcareous substrata (Culberson 1969; Wetherbee 1969; Brodo et al. 2001). While *Cladonia pocillum* was limited to the less acidic soils in Athapap and Spruce Woods Provincial Park (Table 4), *Cladonia pyxidata* was more widespread showing no correlation with pH (Table 7), suggesting that acidic substrata may have been available or the species can tolerate less acidic soils. Species such as *Cladonia chlorophaea* and *Cladonia rangiferina* were found in both acidic and neutral soils, suggesting they may also be more tolerant of pH changes in the substrata than some other species. Whereas some chemotypes of the *Cladonia chlorophaea* complex (*Cladonia cryptochlorophaea*, *Cladonia grayi*, *Cladonia pocillum* (Gilbert 1977; Piercey-Normore 2006), and *Cladonia cariosa* (Culberson 1969)) prefer only calcareous substrata, *Cladonia merochlorophaea* and *Cladonia pyxidata* are known to grow on acidic substrates (Brodo et al. 2001). Patches of soil or substrata with higher pH levels even in a location characterized by low pH levels may explain the presence of these species. For example, *Cladonia merochlorophaea* was reported to grow directly on moss over calcareous soil where the moss provided the acidic substrate for the lichen (Piercey-Normore 2005, 2006, 2008).

Soil characteristics and the CNB hypothesis

The CNB hypothesis (Bryant et al. 1983; Hyvarinen et al. 2003) was not supported by the results of this study. Lichens such as *Cladonia arbuscula* and *Cladonia rangiferina* are mat-forming lichens because they grow acropetally forming a carpet of upright thalli in which the crustose primary thallus disintegrates over time and the upright thallus often grows well above the soil layer. The formation of this mass of vegetative thallus suggests that more carbon was needed to produce a larger mass of thallus (Crittenden 2000). If the CNB hypothesis was supported, the larger mass would have corresponded with fewer secondary metabolites, but this was not the case. Some species with this growth form were more abundant in locations with high organic matter in the soil such as *Cladonia uncialis*, *Cladonia stellaris*, and *Cladonia amaurocraea*, which were predominantly in Sherridon and North Star. However, other species such as *Cladonia arbuscula* and *Cladonia rangiferina* were present in 4 or 5 of the locations, suggesting they could tolerate a wider range of organic matter in the soil. Only *Cladonia arbuscula*, of these mat-forming species, was present in Athapap, which also had significantly higher levels of organic matter than other locations. The larger amount of carbon in the soil with more organic matter may allow for more vegetative growth in these mat-forming species, but all species produced similar numbers of carbon-based secondary metabolites. While the numbers of secondary metabolites in this study showed no support for the CNB hypothesis, it may be hypothesized that the quantity of each secondary metabolite may show more support for the hypothesis.

Soil features may influence the occurrence of some secondary metabolites

The distribution of usnic acid and atranorin, but not fumarprotocetraric acid, may be explained by soil characteristics. The presence of usnic acid was significantly higher in the more acidic sites of Sherridon and North Star (Table 6), where usnic acid may help the lichen tolerate acidic substrata (Hauck et al. 2009*a*). Usnic acid may also serve as an anti-herbivorous substance (Nimis and Skert 2006) or as a sun-screening metabolite (Hauck

Fig. 3. Redundancy analysis (RDA) of the Sherridon Road (S), North Star Road (NS), Athapap Road (A), Spruce Woods Provincial Park (SW), and Sandilands Provincial Forest (SA) locations and percent frequencies of the secondary metabolites, i.e., atranorin (At), barbatic acid (Bar), fumarprotocetraric acid (Fum), grayanic acid (Gra), merochlorophaeic acid (Mer), perlatolic acid (Per), squamatic acid (Sq), thamnolic acid (Thm), usnic acid (Us), and zeorin (Zeo), as constrained by soil features (silt/clay, sand (total, fine, and coarse), gravel, organic matter, soil particle shape (angular and rounded), and pH value). The secondary metabolites (didymic, gyrophoric, lobaric, norstictic, pinastric, salazinic, stictic, and vulpinic acids) were not found to be at significant levels in any of the five locations or were at frequencies <20% and are not included in the figure. The first RDA axis accounts for 62.2% of the total variation and the second axis for 22.8% of the variation.

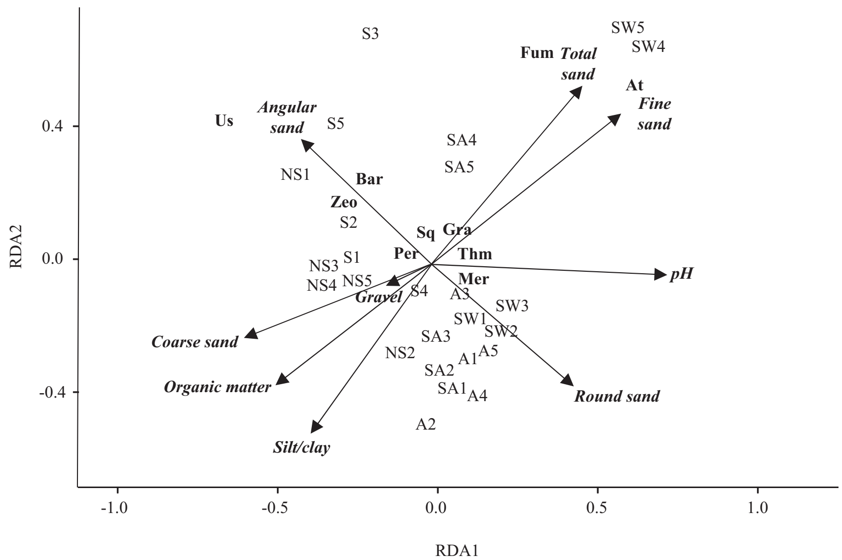
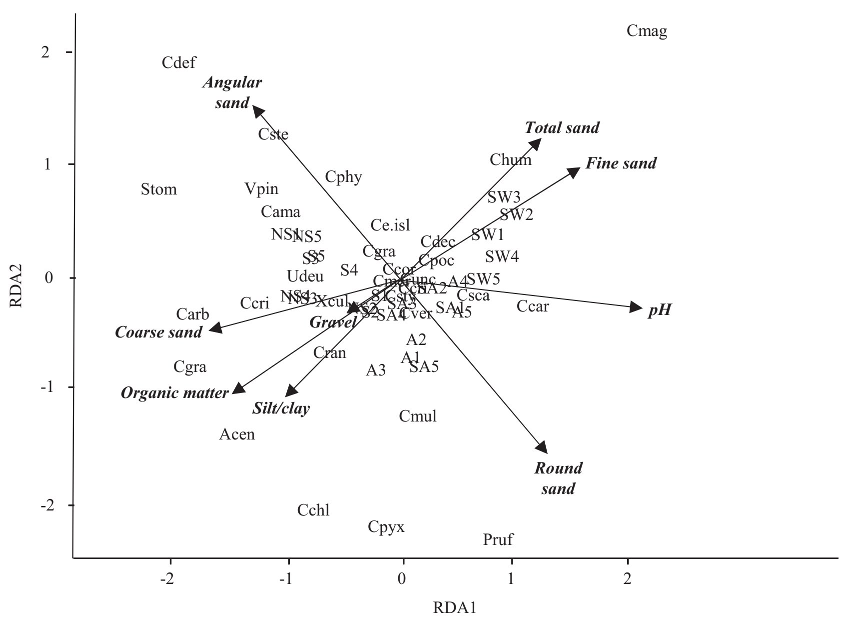


Fig. 4. Redundancy analysis (RDA) of sites and lichen species constrained by soil features (silt/clay, sand (total, fine, and coarse), gravel, organic matter, soil particle shape (angular and rounded), and pH value). Species are represented by the first letter of the genus followed by the first three letters of the species epithet. See Table 7 for a list of the species names. The collection locations are abbreviated as in Fig. 3. The first RDA axis accounts for 44.81% and the second axis for 19.93% of the variation in the data.



2008). Lichen species that produce usnic acid were more dominant on acidic substrata such as *Cladonia amaurocraea*, *Cladonia arbuscula*, *Cladonia cristatella Cladonia deformis*, *Cladonia stellaris*, and *Cladonia uncialis* (Table 3; Hauck et al. 2009*b*; Doering et al. 2016). The production of atranorin and fumarprotocetraric acid may also be affected by sand acidity levels. The presence of atranorin in lichen species in the less acidic sites, such as Spruce Woods Provincial Park and to a lesser extent Athapap (Table 5), may reflect the higher pH levels in the habitat rather

Table 6. Pearson correlation between three main soil characteristics (pH, organic matter, and sand content) and the number of occurrence of secondary metabolites (*n*).

		pH			Organic matter (%)			Sand content (%)		
Secondary metabolite	*n*	R^2	*F*	*P*	R^2	*F*	*P*	R^2	*F*	*P*
Atranorin	141	15.48	22.53	**<0.0001**	–7.30	9.63	**0.002**	11.58	16.10	**0.0001**
Barbatic acid	36	–14.11	20.21	**<0.0001**	2.72	3.44	0.070	9.78e^{-5}	0.01	0.910
Fumarproto-cetraric acid	432	2.40	3.05	0.083	–11.00	15.20	**0.0002**	10.30	14.18	**0.0003**
Grayanic acid	8	0.58	0.72	0.390	1.30	1.60	0.210	2.99	3.79	0.054
Merochloro-phaeic acid	13	0.07	0.09	0.760	0.27	0.34	0.560	0.01	0.02	0.890
Perlatolic acid	20	1.50	1.88	0.170	0.07	0.09	0.760	0.07	0.09	0.760
Squamatic acid	15	–3.21	4.08	**0.045**	0.17	0.21	0.650	0.09	0.11	0.740
Thamnolic acid	3	0.15	0.19	0.670	1.50	1.88	0.170	0.02	2.80	0.090
Usnic acid	185	–25.24	41.50	**<0.0001**	3.60	4.67	**0.032**	2.40	3.04	0.080
Zeorin	46	–12.27	17.20	**<0.0001**	3.30	4.19	**0.042**	0.42	0.52	0.470

Note: Bold values are significant at $p < 0.05$.

Table 7. Pearson correlation between three main soil characteristics (pH, organic matter, and sand content) and the frequency of occurrence of lichen species (*n*).

		pH			Organic matter (%)			Sand content (%)		
Species	*n*	R^2	*F*	*P*	R^2	*F*	*P*	R^2	*F*	*P*
Arctoparmelia centrifuga	25	23.5	7.0847	**–0.0139***	43.1	17.4422	**0.0004****	16.9	4.7060	**–0.0406***
Cladonia amaurocraea	16	40.2	15.4861	**–0.0007****	22.5	6.6996	**0.0164***	4.8	1.1611	–0.2924
Cladonia arbuscula	70	24.3	7.4047	**–0.0122***	16.7	4.6230	**0.0423***	13.2	3.4800	0.0749
Cladonia cariosa	24	16.5	4.5638	**0.0435***	2.9	0.6901	–0.4147	0.29	0.0671	0.7979
Cladonia chlorophaea	65	3.4	0.8168	–0.3755	34.6	12.1679	**0.0020****	23.5	7.1006	**–0.0138***
Cladonia cornuta	37	1.03	0.2406	–0.6284	1.2	0.2907	–0.5950	1.4	0.3320	0.5701
Cladonia crispata	8	13.5	3.5995	–0.0704	0.28	0.0656	0.8001	0.07	0.0175	0.8960
Cladonia cristatella	26	19.5	5.6053	**–0.0267***	2.8	0.6652	0.4231	0.05	0.0020	0.9645
Cladonia decorticata	6	5.7	1.4035	0.2482	3.7	0.8741	–0.3595	6.1	1.4948	0.2339
Cladonia deformis	24	45.9	19.5178	**–0.0002****	3.8	0.9173	0.3481	1.2	0.2874	–0.5970
Cladonia gracilis subsp. *turbinata*	66	42.7	17.1879	**–0.0004****	23.2	6.9482	**0.0148***	14.3	3.8526	**–0.0169***
Cladonia grayi	8	9.2	2.3289	0.1406	4.1	0.9832	–0.3317	5.4	1.3291	0.2608
Cladonia humilis	11	18.5	5.2081	0.0321	15.0	4.0656	–0.0556	37.7	13.9474	**0.0011****
Cladonia magyarica	24	24.5	7.4652	**0.0119***	16.7	4.6328	**–0.0421***	42	16.6716	**0.0005****
Cladonia merochlorophaea	9	0.8	0.1852	0.6709	1.4	0.5679	–0.5679	0.3	0.0735	0.7887
Cladonia multiformis	14	0.01	0.0033	–0.9545	0.53	0.1247	–0.7272	1.2	0.2831	0.5998
Cladonia phyllophora	11	23.4	7.0359	**–0.0142***	1.2	0.3001	0.5891	0.9	0.2111	–0.6502
Cladonia pocillum	4	15.9	4.3614	**0.0480***	1.7	0.4048	–0.5309	3.7	0.8806	0.3578
Cladonia pyxidata	61	0.29	0.0687	0.7955	6.1	1.5001	0.2331	1.5	0.3497	–0.5601
Cladonia rangiferina	24	2.1	0.4928	–0.4897	0.01	0.0027	0.9588	1.2	0.2915	0.5945
Cladonia scabriuscula	15	2.9	0.6834	0.4169	8.3	2.0876	–0.1620	16.7	4.6027	**0.0427***
Cladonia stellaris	17	29.7	9.7325	**–0.0048****	3.7	0.8956	0.3538	1.2	0.2988	–0.5899
Cladonia stygia	5	6.9	1.7141	–0.2034	4.2	1.0196	0.3231	1.1	0.2586	–0.6159
Cladonia uncialis	14	2.9	0.6786	–0.4185	1.9	0.4546	0.5069	1.7	0.4096	–0.5285
Cladonia verticillata	6	0.01	0.0033	0.9547	8.7	2.2149	–0.1503	11.7	3.0625	0.0934
Peltigera rufescens	29	11.07	2.8648	0.1040	4.1	0.9922	0.3296	5.07	1.2226	–0.2803
Stereocaulon tomentosum	31	31.4	10.5529	**–0.0035****	20.3	5.8556	**0.0238***	8.29	2.0792	–0.1628
Umbilicaria deusta	9	22.6	6.7020	**–0.0164***	23.1	6.9313	**0.0149***	4.2	1.0273	–0.3213
Vulpicida pinastri	19	43.1	17.4429	**–0.0004****	13.1	3.4801	0.0749	2.8	0.6744	–0.4199
Xanthoparmelia cumberlandia	8	4.1	0.9856	–0.3311	12.4	3.2572	0.0842	6.2	1.5151	–0.2308

Note: Bold values are significant at $p = 0.05$. *, Significance was determined at $p < 0.05$; **, Bonferroni correction of $p < 0.0005$ (0.05/*X*).

than light or humidity levels in the environment. If light or humidity played a role in the distribution of thalli containing atranorin, the canopy cover should influence their distribution. However, Spruce Woods Provincial Park had the lowest canopy cover and Athapap had the highest canopy cover (Table 3), which does not support the light regulating properties of atranorin to make light available for the photobiont (Melo et al. 2011). However, shrub or herbaceous canopy layers may have played a role in the amount of light reaching the lichens and, therefore, the presence of atranorin in the lichen thallus.

The pH of the soil alone does not seem to influence the type of secondary metabolite produced by lichens in this study. Species containing fumarprotocetraric acid were reported to be more common in acidic sites such as on peat soils (Purvis et al. 1992) or on the bark of conifers (Gauslaa et al. 1998) and the production of fumarprotocetraric acid was thought to increase the tolerance of lichens for acidic substrates (Hauck 2008; Hauck et al. 2009*a*). However, fumarprotocetraric acid was present in all five locations with no significant difference in frequency of occurrence (Table 5). Culberson et al. (1977) reported a correlation with the amount of fumarprotocetraric acid production in the *Cladonia chlorophaea* group with distance from the ocean in North Carolina. The frequency of occurrence of 13 other fumarprotocetraric acid-containing species varied among locations with different pH levels, suggesting that pH was not affecting the production of this compound.

Conclusion

The physical and chemical components of the soil substrate have been previously assumed to be important factors in species assemblages but this is one of the first studies to closely examine soil characteristics relative to species and secondary metabolite occurrence. The finding that many of the species, with some exceptions, were not influenced by soil characteristics, supports the concept that lichens receive their nutrients and moisture from atmospheric sources. However, the few species that showed relationships with soil, highlight valuable implications for stabilizing soils and provide further hypotheses for testing. The CNB hypothesis was not supported in this study because all species produced some secondary metabolites even when carbon was present in the soil. However, the quantification, rather than occurrence, of the secondary metabolites in lichen species may be used in future studies to test the CNB hypothesis more directly. The sand content and shape of the soil particles seemed to have the greatest effect on species composition and the occurrence of some secondary metabolites in this study, but the correlations provide insights that need to be further tested. These novel findings are partially exploratory and draw upon relationships among variables that have not previously been examined.

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